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In vivo generation of highly abundant sequence-specific oligonucleotides for antisense and triplex gene regulation

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ABSTRACT

Antisense and triplex oligonucleotides continue to demonstrate potential as mediators of gene-specific repression of protein synthesis. However, inefficient and heterogeneous cellular uptake, intracellular sequestration, and rapid intracellular and extracellular degradation represent obstacles to their eventual clinical utility. Efficient cellular delivery of targeted ribozymes can present similar problems. In this report we describe a system for circumventing these obstacles and producing large quantities of short, sequence-specific RNA oligonucleotides for use in these gene regulation strategies. The oligonucleotides are generated from a vector containing promoter, capping, and termination sequences from the human small nuclear U6 gene, surrounding a synthetic sequence incorporating the oligonucleotide of interest. *In vivo*, these oligonucleotides are produced constitutively and without cell type specificity in levels up to 5 × 10^6 copies per cell, reach steady-state levels of expression within 9 hours post-transfection, and are still readily detectable 7 days post-transfection. In addition, these oligonucleotides are retained in the nucleus, obtain a 5′ γ-monomethyl phosphate cap, and have an intracellular half-life of approximately one hour. This expression vector provides a novel and efficient method of intracellular delivery of antisense or triplex RNA oligonucleotides (and/or ribozymes) for gene regulation, as well as a cost-effective means of comparing the biological activity arising from a variety of different potential oligonucleotide sequences.

INTRODUCTION

The potential of triplex and antisense oligonucleotides to inhibit selectively protein synthesis from a specified target gene has generated significant enthusiasm for their development as experimental therapeutics. Inhibition of expression of virally-derived proteins (1–3) or endogenously activated oncogenes that contribute to cancer induction and/or progression (4–6) represent two particularly active areas of applied research, although the technology is also a powerful basic science research tool for the functional assessment of specific genes in cellular growth and differentiation (7).

While sufficient evidence indicates that oligonucleotides can cross the multiple cellular membrane barriers needed to reach their intracellular targets (8–10), a growing number of reports suggest that this uptake process is highly inefficient and may exhibit cell-type specificity and heterogeneity (9,10). In addition, imaging studies demonstrate that the typical pattern of oligonucleotide uptake results in oligonucleotide compartmentalization within punctate vesicles believed to be of endosomal origin (8), sequestered from their DNA or RNA targets, and subject to eventual lysosomal fusion and nuclease degradation. Rapid extracellular degradation has also been noted (11). Biological activity is thought to arise from the small fraction of full-length oligonucleotides that either escape from endosomes and rapidly accumulate in the nucleus, or enter the cytoplasm by another process and similarly accumulate intranuclearly. Oligonucleotide/nucleic acid target interactions can thus occur en route to or within the nucleus.

To circumvent these obstacles of extracellular degradation, cellular uptake, and intracellular sequestration, we sought to create a more optimal method for antisense or triplex oligonucleotide delivery, that was sufficiently general for ribozyme delivery as well. The strategy was developed with the following criteria in mind: oligonucleotides should be generated in high yield within the cell nucleus without significant cell type specificity; they should be sufficiently stable, they should contain minimal secondary structure that could mask binding regions, and they should be of a pre-determined and well-defined sequence and length.

To satisfy these criteria, we constructed a chimeric gene containing regulatory regions of the human U6 small nuclear RNA (snRNA) gene and a synthetic double-stranded insert

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bearing the oligonucleotide to be generated. U6 snRNA, which functions normally in conjunction with several small nuclear riboproteins (snRNPs) in the splicing of premature messenger RNA (12), is transcribed in high yield by RNA polymerase III, requires only upstream promoter sequences for initiation, and terminates cleanly upon reaching a string of 4–6 thymidine residues (13–15). Transcript stability is strongly enhanced by 5′ γ-monomethyl phosphate capping (16) which is directed by a 5′ self-complementary hairpin followed by a conserved hexameric AUAUAC sequence (17).

In this report we characterize the abundant production, intranuclear localization, kinetics of expression, capping, and insert-specific stability of transcripts generated from this chimeric gene for potential antisense, triplex, or ribozyme gene regulation strategies.

MATERIALS AND METHODS

Construction of the chimeric gene

The human U6 gene cloned within the SmaI site of pGem1 (Promega, Madison, WI), along with a mutant human U6 gene with bases +25 to +55 replaced by an XhoI restriction site (with A/C substitution at base 24) were generously provided by G. Kunkel and T. Pederson (13). The mutant U6 gene was recloned into a pBluescript (Stratagene, LaJolla, CA) vector to produce single-stranded phage and to allow two site-directed mutations at bases +86 and +88 (T to G and G to A, respectively) to create a unique NsiI restriction site. This plasmid, mU6, was then recloned back into pGEM1, cut with XhoI and NsiI, and religated with a synthetic 38 mer duplex fragment bearing 5′ XhoI and 3′ NsiI compatible overhanging ends (Keystone Laboratories, Menlo Park, CA). Incorporation of the synthetic oligonucleotide was verified by Maxam and Gilbert dideoxy DNA sequencing. The resulting transcript arising from this vector, U6ON, is 25 nucleotides shorter than native U6 RNA (82 vs. 107). Sequences of the upper strand of U6ON and other various inserts are as listed:

U6ON: 5′ TCGACTCCTCTTCCTCCTCCACCTCCTCCTCCCATGCA 3′
U6CTcon: 5′ TCGACCTCCCTTCCCTTCCCTTCCCCTTCCTCCATGCA 3′
U6AS: 5′ TCGACATGAGCATTCATCAGGCGGGCAAGAATGTGATGCA 3′
MU6: 5′ TCGAGCATGGCCCCTGCGCAAGGATGACACGCAATGCA 3′

Cell culture and gene transfection

The human embryonic kidney cell line, 293, and the human breast cancer cell line, MDA453 (ATCC, Rockville, MD), were transfected by electroporation (250 V, 960 μF) with 5 μg to 40 μg of the chimeric gene or promoterless plasmid DNA. Cell viability after transfection ranged from 40–60% (with 293 cells showing slightly higher tolerance to electroporation than MDA453 cells) and was unaltered by increasing gene transfection dosage up to 40 μg/10^7 cells. 293 cells were cultured in minimal essential media with Earle's basic salt solution, 10% fetal calf serum supplemented with 100 U/ml penicillin and streptomycin in 5% CO_2 incubators. MDA453 cells were cultured in Leibovitz L-15 media with 10% fetal calf serum supplemented with 100 U/ml penicillin and streptomycin in the absence of CO_2. Where indicated, cell counts were obtained by Coulter counting.

RNA isolation and Northern blotting

Total cellular RNA was isolated 48 h after transfection by the guanidinium isothiocyanate/cesium chloride centrifugation technique (18). RNA (10–20 μg, as indicated in figure legends) was electrophoresed in 8% polyacrylamide/7 M urea gels, electroblotted onto nylon filters (Amersham, Arlington Heights, IL) in 8 mM Na_2HPO_4/17 mM NaH_2PO_4 buffer for 3 h at 350 mA, and then UV cross-linked onto the filters for 2 minutes. Probes to detect native U6 and the generated oligonucleotide were radiolabeled by random-priming from an 800 base-pair BamHI/EcoRI fragment taken from the original U6 gene or the chimeric gene within pGem1. After membrane hybridization and autoradiography, bands were either quantitated by scanning densitometry or cut from the filter for scintillation counting.

To prepare nuclear and cytoplasmic RNA fractions, transfected cells were electroporated with 10 μg of the chimeric gene and after 48 h, cells were washed twice in phosphate buffered saline (PBS) without calcium or magnesium, and the nuclei extracted by gentle hypotonic lysis (19). After 15 seconds of vortexing and 5 minutes at 4°C, nuclei were pelleted and rewashed in PBS. RNA from the nuclear pellets and the aqueous cytoplasmic fraction were separately extracted in 4 M guanidinium isothiocyanate/cesium chloride as described above.

Transcription arrest

Intracellular stabilities of U6ON and normal U6 were assessed by halting cellular transcription with 10 μg/ml of Actinomycin D (Sigma, St Louis, MO) administered to cell cultures 48 h after transfection. Cells were harvested and total cellular RNA was isolated at 0, 0.5, 1, 2, and 4 h time points after Actinomycin D treatment. Northern blotting was performed to quantitate U6 and U6ON transcript levels.

RNA immunoprecipitation

293 cells were transfected with 20 μg of the chimeric gene or promoterless plasmid DNA, and after 48 h, total cellular RNA was isolated. 20 μg of this RNA was used for immunoprecipitation with 0.5 mg of a 5′ γ-monomethyl phosphate cap-specific antibody, generously provided by R. Reddy. Incubation and precipitation conditions were followed as previously described for this antibody (20).

RNA secondary structure prediction

Proposed secondary structures of the RNA oligonucleotides were obtained using the Martinez algorithm RNAFOLD (21). In all models, loop destabilization was allowed and a maximum 'bulge' size of 30 nucleotides was permitted.

RESULTS

Intracellular generation of RNA oligonucleotides

Figure 1 illustrates schematically the structure of the native U6 snRNA gene, the modifications involved in generating the chimeric gene, and the resulting RNA oligonucleotide transcript, U6ON. As shown, the upstream promoter and enhancer regulatory regions, the initial 25 bp (with A/C substitution at base 24), and the terminal 19 bp of the native U6 gene were retained in the chimeric gene. Mutagenesis and restriction digest removed the remaining native U6 internal sequence in order to create a hybrid of native and synthetic sequences in a gene designed to express any oligonucleotide of interest. As shown in Figure 1c, the transcribed RNA oligonucleotide was designed to retain the original 5′ hairpin, in order to obtain the 5′ γ-monomethyl phosphate cap. This initial sequence is followed by the sequence-

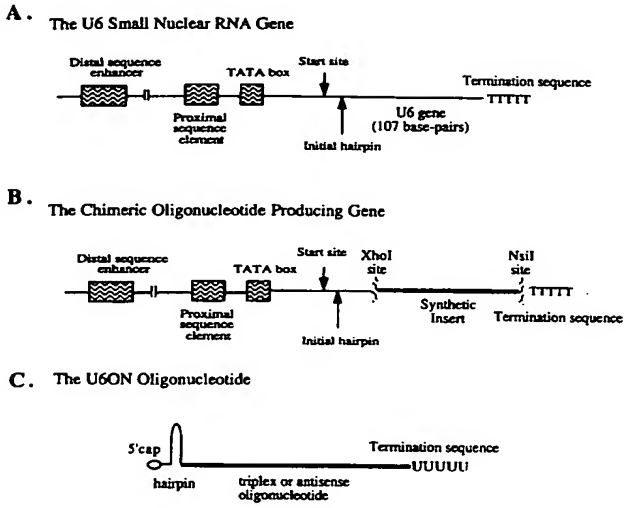

Figure 1. Structure of the U6 snRNA gene, the chimeric U6ON gene and the resulting U6ON transcript. (A) The U6 gene has three critical promoter elements necessary for efficient transcription, a 5' self-complementary hairpin sequence sufficient for capping, and a string of 5 thymidine residues necessary for termination. (B) These elements were retained in the construction of the chimeric gene but its internal sequence was mutated to produce two unique restriction sites for inserting oligonucleotide sequences (bold line). (C) The resulting oligonucleotide may retain the 5' hairpin, followed by the oligonucleotide (bold line) and the native U6 uridine-rich sequence.

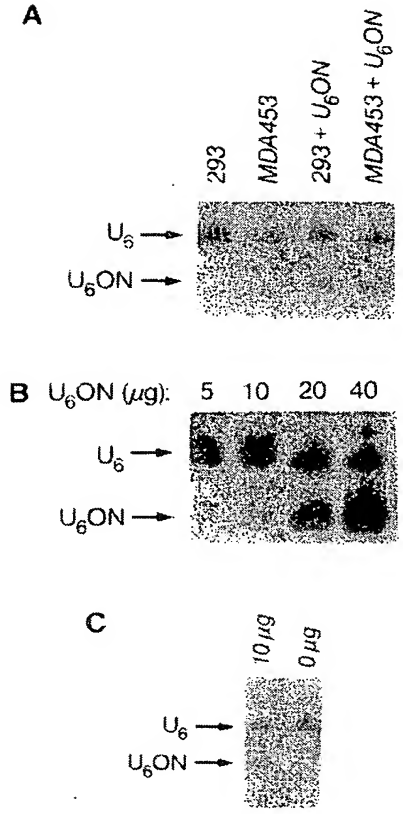

Figure 2. Production and nuclear localization of U6ON. (A) MDA453 and 293 cells were transfected with either 10 μg of the chimeric U6ON gene or 10 μg of promoterless plasmid DNA. Total cellular RNA was isolated 48 h later followed by Northern blotting with both U6 and U6ON radiolabeled probes. (B) MDA453 cells were transfected with increasing quantities of the chimeric gene followed by RNA isolation at 48 h and Northern blotting as described above. All transfections contained 40 μg total DNA, with promoterless plasmid DNA supplementing the chimeric gene as necessary. (C) MDA453 cells were transfected with 10 μg of the chimeric gene and after 48 h, RNA was separated into nuclear and cytoplasmic fractions. The nuclear fraction shown above contained the U6ON transcript along with the native U6 snRNA. All Northern blots were generated from 10 μg of RNA loaded/well.

specific oligonucleotide and the native U6 uridine-rich 3' terminus. The total length of the resulting transcript is a function of the synthetic oligonucleotide inserted—for the experiments described in this report, we inserted a 38 bp duplex yielding a U6ON of 82 nucleotides.

Figure 2a demonstrates the intracellular generation of this sequence-specific RNA oligonucleotide, U6ON, in two different human cell lines, MDA453 and 293, following transfection with 10 μg of the chimeric gene. No cell-type specificity in production has been observed in any of 5 different human cell lines transfected with the chimeric gene. However, transcript levels vary in accordance with the amount of chimeric gene transfected within the range of 5 to 40 μg plasmid DNA per 107 cells.

Figure 2b illustrates that within this 8-fold range of transfected gene dosage, a near 100-fold linear variation in U6ON transcript levels is observed. Using native U6 RNA levels (known to be present at roughly 0.5 ×106 copies per cell (22)) as a marker, we performed densitometry to compare the U6ON bands at each transfection level with the U6 band at the 5 μg gene transfection level. (Previous results have shown that native U6 transcript levels do not vary upon transfection with 0, 5, or 10 mg U6ON gene transfection levels, but do show transient decreases in transcript levels after transfection with 20–40 μg of the U6ON gene). From this analysis, we estimate steady-state intracellular U6ON transcript levels to range from 5 ×10⁴ to 5 ×10⁶ copies/cell (at 48 h post-transfection), depending upon quantity of gene transfected. If nuclei are assumed to be spherical and to have an average diameter of 10 mm, these values correspond to an intranuclear (see Fig. 2c) concentration ranging from 160 μM to 16 μM. (These calculations assume an even distribution of the U6ON gene throughout the electroporated cell population, as is found for the U6 gene. Errors resulting from this assumption may lead to higher actual intracellular transcript concentrations.)

As shown in Figure 2c, when RNA from gene transfected or mock transfected MDA453 cells is separated into nuclear and cytoplasmic fractions, U6ON is found predominantly in the nuclear fraction, along with native U6. U6ON could not be detected to any significant extent in the cytoplasmic fraction. Moreover, the relative ratio of U6 to U6ON found in the nuclear fraction mirrors the ratio found in total cellular RNA samples.

Kinetic analysis of U6ON expression

Figure 3 illustrates the rapid production, steady-state levels, and decaying expression of U6ON in 293 cells and MDA453 cells. In Figure 3a, analysis over the first 48 h post-transfection shows

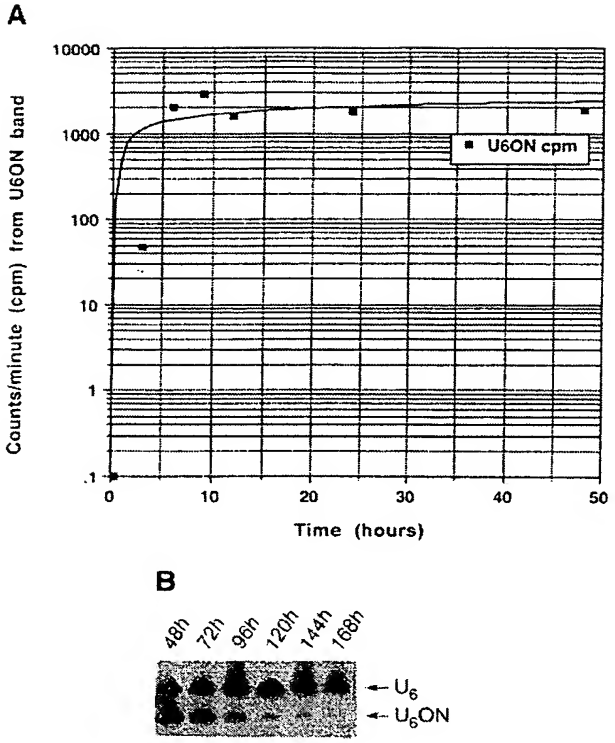

Figure 3. Kinetics of U6ON expression. (A) 293 cells were transfected with 20 μg of the chimeric gene and total cellular RNA was isolated at 0, 3, 6, 9, and 12 h time points. After Northern blotting (10 μg RNA added/well) and autoradiography with a U6ON radiolabeled probe, the U6ON bands were cut from the filter and scintillation counted. (B) MDA453 cells were transfected with 20 μg of the chimeric gene and total cellular RNA was isolated at 48, 72, 96, 120, 144, and 168 h time points. Northern blotting followed with 20 μg of RNA added/well.

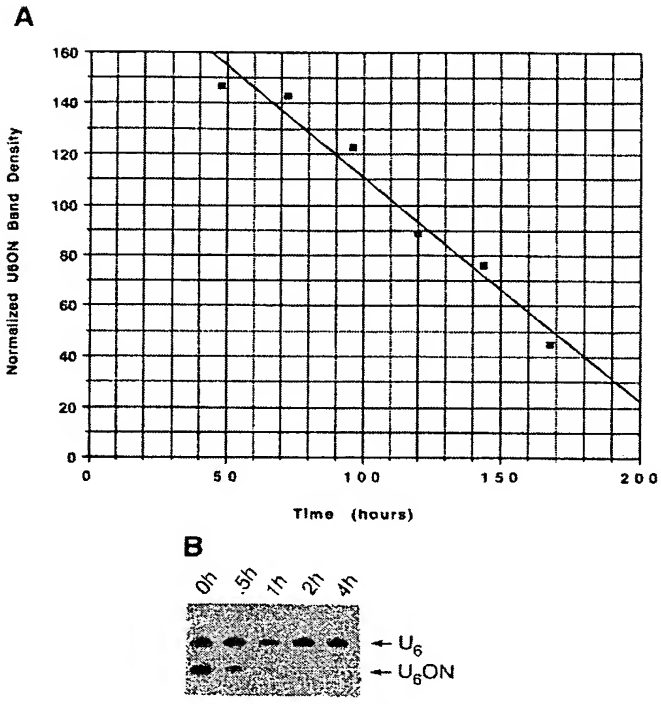

Figure 4. Intracellular stabilities of the chimeric gene and the U6ON transcript. (A) Cell counting in parallel with the RNA isolations of Figure 3b allowed the U6ON band densities to be normalized to account for the dilutional effects of cell division. Normalized band densities were plotted as a function of time to determine the rate of chimeric gene degradation (or inactivation). (B) 293 cells were transfected with 5 μg of the chimeric gene and after 48 h, cellular transcription was halted by a 10 μg/ml treatment of Actinomycin D. At 0, 0.5, 1, 2, and 4 h time points, RNA was isolated. Northern blotting (20 μg RNA/well) with U6 and U6ON radiolabeled probes, followed by densitometry of the U6ON bands, allowed for the determination of U6ON half-life.

that U6ON expression from the chimeric gene begins within 3 h post-transfection, and reaches steady-state levels in less than 10 h. Between 12 h and 48 h post-transfection, steady-state U6ON levels are constant. The Northern blot shown in Figure 3b demonstrates the decline in U6ON transcript levels out to 168 h post-transfection where production is diminished but still readily detectable.

Intracellular stabilities of the chimeric gene and the U6ON transcript

Intracellular stability of the transfected gene was estimated under the assumption that the observed decline in U6ON transcript levels between 48 h and 168 h (Figure 3b) arises predominantly from two major causes: plasmid degradation (or functional inactivation) and the dilutional effect of cell division (given equal RNA loading per lane). The dilutional effect of cell division was accounted for by cell counting in parallel with RNA isolation from 48 h to 168 h, and normalizing the Northern blot density values by these cell counts. (Normalized band density = [absolute cell count/cell count at 48 h] * raw band density.) The rate of plasmid degradation (or inactivation) was then estimated as the amount of time required for transcript levels to diminish by 50% from steady-state (48 h) levels. Cell counting revealed a 38 h average doubling time from 48 h to 120 h, after which time, cell confluence was reached. From 120 h to 168 h, absolute cell counts declined slightly. Figure 4a demonstrates the fairly constant plasmid degradation (or inactivation) rate after this normalization procedure, suggesting a zero-order decay process with plasmid half-life determinations dependent on the initial (48 h) plasmid levels. Thus, given the above assumptions and constraints, after a 20 μg transfection of the chimeric gene into 107 cells, approximately 50% of the chimeric gene remains functional after 96 h (4 days). Variations of this estimate in different cell types would be expected.

In contrast, the intracellular half-life of the U6ON transcript was directly measured by halting cellular transcription with Actinomycin D treatment 48 h after transfection with 5 μg of the chimeric gene, and monitoring the decay of intensity from the U6ON band in Northern blots. The native U6 band was also monitored as a control since its intracellular half-life is known to be 16–24 h (22, 23) Figure 4b demonstrates the decline in U6ON band intensity between 0 h and 4 h following transcription arrest, indicating an intracellular half-life of approximately 1 h after quantitation by densitometry. This analysis also confirms

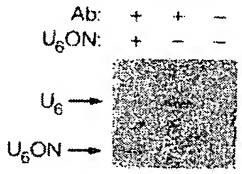

Figure 5. Immunoprecipitation of U6ON. Total cellular RNA samples isolated after a transfection with 20 μg of U6ON in 293 cells were immunoprecipitated with 0.5 mg of a U6 cap-specific antibody as previously described (20). Each immunoprecipitation required 20 μg of initial total cellular RNA. Immunoprecipitated RNA was used for Northern blotting with U6 and U6ON radiolabeled probes as described in Materials and Methods.

the prolonged stability of native U6. Similar U6ON half-life values were obtained when the experiment was repeated with 10 μg and 20 μg gene transfections. Such results indicate that increases in absolute U6ON steady-state transcript levels do not affect U6ON half-life determinations, consistant with a first-order process of transcript degradation.

U6ON obtains a 5′ γ-monomethyl phosphate cap

To determine whether the retention of the capping signal of native U6 in the U6ON gene allowed for the production of capped U6ON transcripts, we performed RNA immunoprecipitations with a 5′ γ-monomethyl phosphate cap-specific antibody (20). This antibody has previously been shown to be specific for U6, 7sk, and several other unidentified transcripts which contain this unique 5′ cap. Figure 5 illustrates that the U6ON transcript is specifically recognized and immunoprecipitated by this antibody after a 20 μg gene transfection in 293 cells, despite an A/C substitution at base 24. (The relative decline in native U6 transcripts immunoprecipitated in the presence of U6ON may be attributed to limiting levels of the antibody, a transient decrease in U6 transcript levels at this higher transfection dose, or competition with U6 RNA for capping enzyme/s and/or substrates).

Transcript stability may depend upon the oligonucleotide insert sequence

As seen in Figure 5, immunoprecipitating total cellular RNA with a U6 cap-specific antibody confirmed that the U6ON obtains the 5′ γ-monomethyl phosphate cap structure found on native U6 RNA. As capping has been previously shown to augment greatly transcript stability and to be dependent on a stable 5′ self-complementary hairpin (16, 17), we sought to determine if insert sequences which favor disruption of the initial 5′ hairpin for a longer and more stable stem-loop secondary structure reduce overall transcript stability, and thus steady-state transcript levels.

Figure 6a demonstrates the conformational output of the RNA secondary structure prediction algorithm RNAFOLD (21), given two different oligonucleotide insert sequences, U6CTcon and U6AS. Despite the same initial nucleotide sequence derived from native U6 in both transcripts, the expected ability to retain the 5′ initial hairpin within this sequence differs as a result of the downstream insert sequence. (The overall structure and energy values obtained for U6ON and mU6, mirror U6CTcon and U6AS, respectively.) Using this structure prediction program, we then designed and constructed a variety of chimeric genes

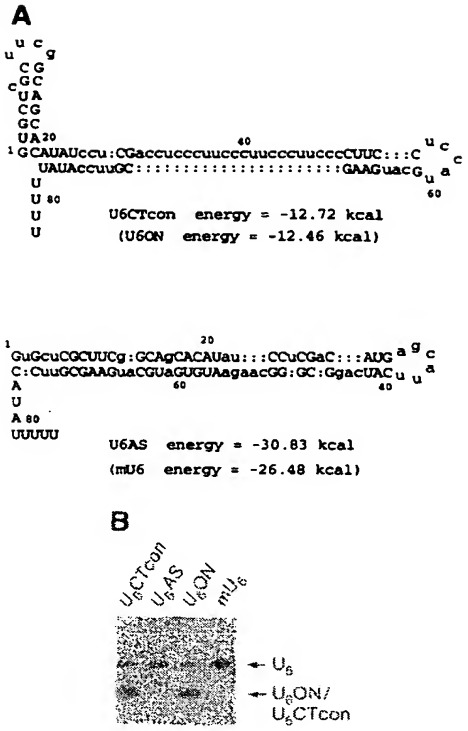

Figure 6. Insert sequence-specific effects on transcript secondary structure and intracellular transcript levels. (A) RNA Secondary structures and associated energies were predicted for two different constructs, U6CTcon and U6AS, using the program RNAFOLD (21). Upper case letters refer to base-pairings, lower case letters refer to mismatches and colons refer to bulged regions. The energies of U6ON and mU6, which were found to have similar structural profiles to U6CTcon and U6AS, respectively, are given in parentheses. (B) RNA secondary structure was predicted for 4 different oligonucleotide transcripts and the corresponding chimeric genes were constructed. 20 μg of the chimeric genes were transfected into MDA453 cells, followed by Northern blotting (20 μg RNA added/well) 48 h later with a U6 probe and a probe for each of the possible RNA transcripts.

which generate transcripts that are predicted to prefer one conformation over the other.

Figure 6b demonstrates that when the algorithm predicts that the 5′ hairpin is disrupted by downstream secondary structure (as in U6AS and mU6), steady-state transcript levels are drastically reduced. Only at very long film exposures (6 days) can the bands corresponding to mU6 and U6AS be observed.

Ten chimeric gene constructs have been created to test the hypothesis that the insert sequence can affect intracellular transcript stability and thus steady-state transcript levels by interfering with the formation of the initial 5′ hairpin (6 which the algorithm predicts to retain the initial 5′ hairpin, and 4 which the algorithm predicts to disrupt the initial 5′ hairpin). Of these 10 constructs, 8 conform to the pattern of expression and stability shown in Figure 6b in both MDA453 cells and 293 cells. The two constructs which did not conform were designed to generate stable RNA transcripts, but upon transfection and Northern blotting were found to generate unstable transcripts. All constructs

designed to generate unstable transcripts gave rise to unstable transcripts. These disparities may arise from limitations in predicting a preferred RNA state from two competing states *in vivo*. Alternatively, capping and/or stability of these different transcripts may be governed by a more complex set of principles.

DISCUSSION

In this report we describe the design and construction of a vector capable of generating a large intracellular pool of short triplex or antisense RNA oligonucleotides in order to circumvent the many obstacles of cellular uptake, sequestration, and degradation of extracellularly-added oligonucleotides. Levels of production of this U6ON oligonucleotide can rival and even exceed those of the native U6 snRNA ($5 \times 10^4 - 5 \times 10^6$ copies/cell) and, like native U6 RNA, U6ON is capped and remains intranuclear in concentrations which may range from 160 μM to 16 mM. U6ON production occurs rapidly upon transfection and can still be detected up to one week after transfection, as 50% inactivation of the parent plasmid from steady-state requires approximately four days. This long-lived production suggests that in slowly growing cell populations, longer time points (i.e. longer than the typical 48–72 h) may be used for measuring a biological response from a transient transfection of the chimeric gene. In addition, this long-lived production may allow for the detection of biological effects of antisense or triplex oligonucleotides after transient transfection, even when the target mRNA and/or protein is fairly stable.

The half-life of the individual U6ON transcript is estimated to be 1 hour; however, transcript stability may be dependent upon the sequence of the oligonucleotide insert. We put forth the hypothesis that the sequence of the insert may affect the ability of the transcript to retain the initial 5′ hairpin structure, and thus the ability to obtain a 5′ cap. In support of this hypothesis, correlations have been observed between RNA secondary structure predictions and experimental determinations of transcript levels containing different oligonucleotide insert sequences. When the algorithm predicted the loss of the 5′ hairpin, dramatic decreases in transcript levels were seen experimentally after electroporation and Northern blotting.

Low transcript levels must reflect either a decrease in production or an increase in degradation. As the U6 gene has consistantly been shown to require only upstream promoter sequences for transcription (13), we believe that the low transcript levels seen with some oligonucleotide insert sequences cannot be due to a decrease in production, and therefore are due to an increase in degradation. U6 stability has been attributed primarily to its 5′ cap and its extensive hybridization with U4 (23). As all of the chimeric genes have the U6/U4 hybridization regions deleted, we are led to believe that differences in stability are due to the presence or absence of a 5′ cap. Finally, correlations between our modelling studies and our experimental data point toward the retention of a 5′ hairpin structure as a means of linking oligonucleotide sequence, retention of the 5′ cap, transcript stability, and thus, absolute transcript levels. Consequently, in the design of an oligonucleotide insert, the overall secondary structure of the RNA transcript may have importance in determining transcript stability and steady-state transcript levels.

There are a variety of potential applications for a system which generates sequence-specific short RNA's in high yield within the cell nucleus. For example, antisense oligonucleotides can be generated intracellularly in levels several orders of magnitude greater than typical sense mRNA molecules and far greater than antisense mRNA generated by more traditional vectors that rely on RNA polymerase II for transcription. In addition, the ability to produce short transcripts minimizes the chances that the binding region for a targeted biological effect is masked by secondary structure which can occur with much larger antisense mRNA transcripts that do not have pre-determined length or sequence.

While the number of reports citing successful antisense RNA-mediated inhibition of protein synthesis are numerous and continue to accumulate, adequate delineation of the exact mechanism of its effect is lacking. The formation of duplex regions of RNA does not serve as a substrate for RNase H, an enzyme which cleaves RNA in DNA/RNA hybrids and thought to play a major role in the effectiveness of antisense DNA. However, an RNA oligonucleotide may have increased binding affinity for its target over its DNA counterpart which may translate into an increased ability to block ribosomal assembly or progression. Or alternatively, the effect may be due to the recently described unwinding/modifying activity of RNA duplexes found ubiquitously in mammalian cells (24). This activity has been shown to lead to the deamination of adenosine residues to inosine residues which are subsequently miscoded by the translational machinery as guanosine residues. Thus, regions of duplex RNA might alter RNA degradation rate by its unwinding activity, or produce nonfunctional proteins by its modifying activity. The determination of the true mechanism of antisense RNA effect will ultimately guide the use of this delivery system for antisense purposes.

However, this system may also provide a means for the generation of intranuclear triplex RNA oligonucleotides. Pyrimidine-rich triplex RNA oligonucleotides which bind in a parallel fashion with respect to the corresponding purine strand of a homopurine/homopyrimidine duplex (25), while still maintaining a problematic pH-dependence, have a greatly increased binding affinity over their triplex DNA oligonucleotide counterparts (26). The high concentration of a triplex RNA oligonucleotide which is both generated and retained in the nucleus in vast excess over its DNA duplex target may drive triplex binding to a critical element on a gene promoter, and block subsequent gene expression.

In addition to antisense and triplex oligonucleotides, this chimeric gene may also prove useful in generating longer length ribozyme transcripts for use in binding and cleaving target mRNA. Combinations of triplex and antisense oligonucleotides as well as ribozymes targeted to a single gene may also yield synergistic approaches to the selective repression of gene expression.

Other potential uses include the quenching of specific single-stranded nucleic acid binding proteins by short RNA sequences, or generating self-complementary RNA hairpins that can mimic known DNA binding consensus sequences, thus quenching specific DNA binding transcription factors. While still largely theoretical, these potential applications rely on the nuclear localization of abundant and sufficiently stable oligonucleotides with short and fully defined sequences to allow for reasonable approximation of secondary structure. With ever-increasing potential applications of oligonucleotides, this novel technique for generating sequence-specific RNA oligonucleotides intracellularly offers a powerful new tool for research on nucleic acid-based strategies of selective gene repression.

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